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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Helicase-Dependent Amplification of Circular Nucleic Acids

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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Respectfully submitted,

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REGISTRATION NO. 37,008 *(If appropriate)*

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In re application of: Kong, Huimin

Application No.: not yet assigned
Filed: herewith
For: Helicase-Dependent Amplification of Circular Nucleic Acids

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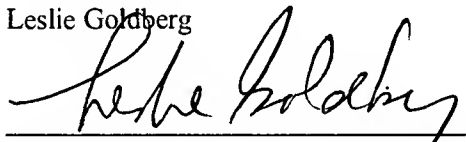

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Docket No. : NEB-245

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

Inventors: Huimin Kong

Title: Helicase-Dependent Amplification of Circular Nucleic Acids

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HELICASE-DEPENDENT AMPLIFICATION OF CIRCULAR NUCLEIC ACIDS

FIELD OF INVENTION

Embodiments of this invention relate to methods for amplifying circular nucleic acids using a DNA helicase, a DNA polymerase, and optional accessory proteins such as single stranded DNA binding protein.

BACKGROUND

DNA amplification is becoming a basic technique in biological studies. So far the most widely used amplification method is the polymerase chain reaction (PCR), which is a target amplification method (See for example, U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159). In a PCR reaction, a thermostable DNA polymerase is used to amplify a specific sequence defined by two primers. Denaturation of duplex DNA is achieved by cycled heating in a thermocycler.

One disadvantage of PCR is its requirement of thermo-cycling. An alternative scheme for nucleic acid amplification is isothermal target amplification. In a Strand Displacement Amplification (SDA) reaction, double strand DNAs are nicked by a restriction endonuclease and then extended by a nuclease-deficient DNA polymerase. Meantime, the strand displacement activity of the DNA polymerase displaces the downstream strands in the duplex, which serves as a template for an antisense reaction and vice versa, leading to exponential

amplification of the target DNA (See, for example, U.S. Patent Nos. 5,455,166 and 5,470,723).

Another isothermal DNA amplification method is Rolling Circle Amplification (RCA). When one primer was used, a linear RCA amplification produces a continuous sequence of tandem copies of the circle DNA templates due to the strong strand displacement activity of a DNA polymerase (Fire, A. and Si-Qun Xu, *Proc. Natl. Acad Sci. USA* 92:4641-4645 (1995); Lui, *et al.*, *J. Am. Chem. Soc.* 118:1587-1594 (1996)). When two primers are used, initial linear RCA amplification is coupled with a cascade of strand displacement reactions that lead to an exponential amplification of circular DNA called hyperbranched RCA (HRCA) (Lizardi, *et al.*, *Nature Genetics* 19:225-232 (1998)) or ramification amplification (Zhang, *et al.*, Gene 211, 277-285 (1998)). Dean et al. (2001) invented multiply-primed RCA, in which random hexamers were used to amplify the whole plasmid by phi29 DNA polymerase (Dean et. al., *Proc. Natl. Acad Sci. USA* 99:5261-5266 (2002)). The multiply-primed RCA can achieve up to 10,000-fold amplification from plasmid DNA, colonies, or plaques. However, the final amplification product is highly branched and viscous, which is difficult to pipette.

The multiply-primed RCA has been developed into a whole plasmid amplification kit using random hexamers (Templiphi™, Amersham Biosciences, Piscataway, NJ). DNA templates are first heat-denatured to provide single stranded DNA for hexamers to anneal. Phi29 DNA polymerase then extends from hexamer-primed sites and displaces the

downstream nontemplate DNA, which becomes single stranded DNA templates for further amplification. Templiphi™ system generates large amounts of product (1-3 micro-gram) from as little as 0.01 ng of purified plasmid DNA or a single bacterial colony. The resulting amplification product can be directly used for sequencing (Nelson et al., *Biotechniques* Suppl: 44-7 (2002)).

SUMMARY OF THE INVENTION

Embodiments of the invention include the following:

(1) A method for amplifying an entire circular nucleic acid as well as a target sequence within the circular molecule comprising:

(a) providing the circular nucleic acid that may contain the target sequence;

(b) contacting the circular nucleic acid annealing two sequence specific oligonucleotide primers flanking the target sequence;

(c) synthesis of DNA from the primers by means of rolling circle amplification on one nucleic acid strand and strand-displacement amplification on the complementary strand, wherein the DNA synthesis is depend on a DNA polymerase, a helicase preparation, and dNTPs.

(2). A method according to (1) wherein amplification is isothermal.

(3). A method according to (1) wherein the circular nucleic acid in step (a) is a circular DNA.

(4). A method according to (3), wherein the circular DNA of step (a) is a double stranded DNA.

(5). A method according to (1), wherein the circular DNA of step (a) is a single stranded DNA.

(6). A method according to (1), wherein the circular has a size in the range of about 50 bp to 500 kb.

(7). A method of (1), wherein the oligonucleotide primers are a pair of oligonucleotide primers wherein one primer hybridizes to 5′-end and one primer hybridizes to 3′ end of the target nucleic acid to be selectively amplified. More than two primers may be included.

(8). A method according to (1), wherein the oligonucleotide primers have a length and a GC content so that the melting temperature of the oligonucleotide primers is higher than 25°C.

(9). A method according to (1), wherein the DNA polymerase is selected from the T7 or T7-like DNA polymerase and its exonuclease-deficient forms such as Sequenase.

(10). A method according to (9), wherein the DNA polymerase lacks 5′ to 3′ exonuclease activity.

(11). A method according to (9), wherein the DNA polymerase preferably possesses a strand displacement activity.

(12). A method according to (1), wherein the helicase preparation comprises one or more DNA helicase plus other components, such as cofactor and accessory proteins (if any), required for unwinding of DNA.

13. A method according to 12, wherein the helicase preparation comprises a plurality of helicases.
14. A method according to claim 12, wherein the helicase preparation comprises a processive helicase, such as a hexameric replicative helicase.
15. A method according to 12, wherein the helicase preparation comprises a T7 DNA helicase, such as T7 gene 4B protein.
16. A method according to 12, wherein the helicase preparation comprises a helicase and a cofactor for the helicase such as nucleotide triphosphate (NTP) or deoxynucleotide triphosphate (dNTP).
17. A method of 16, wherein the cofactor for T7 helicase is dTTP, ATP or dATP and their concentrations are in the range of about 0.1-100mM.
18. A method according to 12, wherein the helicase preparation comprises additional accessory proteins.
19. A method according to 12, wherein accessory protein for T7 gene 4B helicase is a single strand DNA binding protein.
20. A method according to 19, wherein the single strand DNA binding protein is T7 gene 2.5 protein SSB.
21. A method according to 12, wherein the helicase preparation comprises T7 4B helicase, dTTP, and T7 gene 2.5 protein SSB.
22. A method according to 1, wherein steps (a)- (c) are performed at a substantially single temperature in the range of about 15°C-75°C.

23. A method according to 1, wherein steps (a)-(c) are performed at room temperature (say 25°C).

24. A method according to 1, wherein the target circular DNA is purified from a cell culture.

25. A method according to 1, wherein the target circular DNA is in a cell culture.

26. A method according to 1, wherein the target circular nucleic acid is synthesized.

27. A whole plasmid amplification kit, comprising: a helicase preparation, a DNA polymerase, and instructions for performing helicase-mediated amplification of circular nucleic acids according to claim 1.

28. A nucleic acid amplification kit according to 27, comprising: T7 gene 4 helicase, T7 SSB, dTTP or ATP, T7 Sequenase and instructions for performing amplification according to claim 1.

29. A method according to 1, wherein the nucleic acid is in the size range of about 50 nucleotides to 500,000 nucleotides.

30. A method for determining whether a helicase is suited for c-HDA, comprising;

(a) preparing a helicase preparation comprising the helicase, an NTP or dNTP, a buffer, wherein the buffer has a pH in the range of about pH 6.0- 10.0, a concentration of salts such as NaCl or KCl or NaAcetate or KAcetate in a concentration range of 0-200 mM, and Tris-acetate or Tris-HCl and optionally one or more of a single stranded binding protein and an accessory protein;

(b) adding a circular nucleic acid, oligonucleotide primers and a DNA polymerase to the helicase preparation.
(c) incubating the mixture at a temperature between about 15°C and 75°C for up to several days; and
(d) analyzing the DNA on an agarose gel to determine whether selective and exponential amplification has occurred.

31. A method according to 30, further comprising optimizing the conditions of c-HDA by varying the concentration of any or each of: the helicase; the single stranded binding protein; the accessory protein; the NTP or dNTP; the salt concentration; the pH; and varying the buffer type; the temperature; the time of incubation and the length of the target nucleic acid.

32. A DNA amplification kit for amplifying circular nucleic acids. The kit composes: a DNA polymerase, a helicase preparation, a set of four deoxynucleotides, reaction buffer and instructions for performing circular Helicase Dependent Amplification according to 1.

33. A nucleic acid amplification kit according to 32, wherein the helicase preparation may include one or more helicases, and the helicase preparation may further comprise one or more cofactors as well as accessory proteins.

34. A nucleic acid amplification kit according to 33, wherein the accessory protein is a single stranded DNA binding protein.

35. A nucleic acid amplification kit according to 22, comprising: T7 gene 4 helicase, a cofactor such as dTTP for the helicase, T7 gene 2.5 single strand binding protein, dTTP (1 to 50 mM), a T7-like DNA polymerase, the reaction buffer, dNTPs, and instructions for performing amplification according to 1.

36. A method to detect pathogens that harbor DNA plasmids; that uses circular DNA amplification method to amplify and detect DNA plasmids associated with pathogens for the purpose of diagnostics.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic Diagram of Circular Helicase-dependent Amplification (c-HDA). A single stranded circular template is shown as a dotted circle. An antisense primer (solid lines with arrow head) anneals to the template and rolling circle amplification produces a concatemer of the template. Multiple sense primers (dotted lines with arrow head) anneal to the concatemer and extended by the DNA polymerase. As the polymerization runs into a downstream primer extension product, the Helicase/DNA polymerase complex will displace the nontemplate strand. Multiple rounds of displacement and

polymerization produce specific target DNA defined by two primers and multimers of the DNA template.

Figure 2A. c-HDA amplification and its essential components. Lane 1, A 50 µl reaction was set up by mixing 1 unit sequenase (USB), 6 µg T7 SSB, 200 ng T7 4B helicase, 10 ng plasmid pREP, 50 nmole dNTP, 500 nmole dTTP, 20 pmole S1224, 20 pmole s1233, and 1x c-HDA buffer (35mM Tris.Acetate pH7.5, 5 mM DTT, 11 mM MgAcetate). After an overnight incubation at 25°C, 5 µl of amplified product was separated on a 1% agarose gel; lane 2, same reaction as lane 1 but without sequenase; lane 3, same reaction as lane 1 but without T7 gene 4B helicase; lane 4, same reaction as lane 1 but without T7 gene 2.5 SSB; lane 5, same reaction as lane 1 but without T7 4B and T7 SSB; lane 6, same reaction as lane 1 but replacing T7 gene 2.5 SSB with T4 gene 32 SSB.

Figure 2B. Pulse-field Gel electrophoresis of c-HDA products. DNA samples were separated on a 1% low melt agarose in a contour clamped homogenous electric field at 6 volts/cm. The gel was run with a switch time of 1.5 to 11 seconds at 14°C for 16.5 hrs. M, low range PFG marker (NEB); lane 1, template plasmid pREP as the starting material; lane 2, The c-HDA product amplified from 10 ng of plasmid pREP.

Figure 2C. Comparison of c-HDA reactions with or without a prior heat-denaturation step. Lane 1, One-step c-HDA: 50 µl reaction were set up by mixing 1 unit sequenase (USB), 6 µg

T7 SSB, 200 ng T7 4B helicase, 10 ng plasmid pREP, 50 nmole dNTP, 500 nmole dTTP, 20 pmole S1224, 20 pmole s1233, and 1x c-HDA buffer (35mM Tris.Acetate pH7.5, 5 mM DTT, 11 mM MgAcetate). The reaction was incubated at 25°C overnight. Five µl product was separated on a 1% agarose gel. Lane 2, Two-step c-HDA: plasmids, primers, and c-HDA buffer were first denatured at 95°C for 3 min. Then the rest c-HDA components were added and incubated at 25°C overnight. Five µl product was separated on a 1% agarose gel.

Figure 3A. Diagram of plasmid pREP. The relative positions of primers and restriction enzyme sites are indicated.

Figure 3B. Restriction enzyme digestion on the c-HDA products from pREP. An aliquot of c-HDA product from template pREP was used in each digestion. Lane 1, Acc65I digestion on pREP; Lane 2, SacI digestion on pREP; Lane 3, SacI and XhoI double digestion on pREP; Lane 4, pREP plasmid; Lane 5, Acc65I digestion on the c-HDA product; Lane 6, SacI digestion on c-HDA product; Lane 7, SacI+XhoI digestion on HDA product; Lane 8, the c-HDA reaction product; M: 2-log DNA ladder (NEB).

Figure 4. Direct sequencing of c-HDA products from pREP plasmids. A 50 µl c-HDA reaction was set up as described as in one-step c-HDA (FIG 2C, lane 1). After an overnight reaction, 1 µl product was directly sequenced using an ABI sequencer. A, part of the sequencing trace data using primer

S1224; B, part of the sequencing trace data using a *Rep*-specific primer.

Figure 5. Exponential c-HDA amplification requires two primers. A 50-µl reaction was set up as in one-step c-HDA except for the difference in primers. Lane 1, the reaction contained 20 pmole each of primer S1224 and primer S1233; lane 2, the reaction contained 20 pmole of primer S1224; lane 3, the reaction contained 20 pmole primer S1233. M: 2-log DNA ladder (NEB).

Figure 6. c-HDA amplification from a colony of *E. coli* cells containing plasmid pREP. Lane 1 is the positive control which contains 10 ng of purified plasmid pREP in resuspension buffer. A freshly grown colony containing pREP was resuspended in 20 µl resuspension buffer (20 mM Tris.Acetate pH7.5). Various amounts of cell suspensions were added in parallel reactions: lane 2 = 1 µl, lane 3 = 2 µl, lane 4 = 3 µl, lane 5 = 4 µl, and lane 6 = 5 µl. Cell suspensions were heated for 3 min at 95°C and then cooled to 25°C. A 45 µl mix containing 1 unit sequenase (USB), 6 µg T7 SSB, 200 ng T7 4B helicase, 50 nmole dNTP, 500 nmole dTTP, 20 pmole each of primer S1224 and primer S1233, and 1x c-HDA buffer was added to each tube and incubated at 25°C for 6 hrs. Ten µl products were separated on a 1% agarose gel. M: 2-log DNA ladder (NEB).

Figure 7A. Amplification of a 10-kb fragment from pTopo10k.

Lane 1, A 50 µl reaction was set up by mixing 10 ng plasmid pTopo10k, 1 unit sequenase (USB), 6 µg T7 SSB, 200 ng T7 4B helicase, 50 nmole dNTP, 500 nmole dTTP, 20 pmole each of primer S1224 and primer S1233, and 1x c-HDA buffer. After an overnight reaction, 5 µl product was separated on a 1% agarose gel. M: 2-log DNA ladder (NEB).

Figure 7B. Pulse-field Gel electrophoresis of c-HDA products from pTopo10k. DNA samples were separated on a 1% low melt agarose in a contour clamped homogenous electric field at 6 volts/cm. The gel was run with a switch time of 1.5 to 11 seconds at 14°C for 16.5 hrs. Lane 1, template plasmid pTopo10k; lanes 2 and 3, The c-HDA amplification product from pTopo10k. M, Mid-range PFG marker (NEB).

Figure 8. Sequence listing for Primers 4B51, 4B31, 2551 and 2531.

DETAILED DESCRIPTION OF EMBODIMENTS

In an embodiment of the invention, a method of circular-Helicase-Dependent Amplification (or c-HDA) is provided for amplifying nucleic acids from a circular DNA template. This system combines a DNA polymerase and a helicase preparation to amplify a specific sequence as well as the whole circular DNA molecule from a circular DNA template such as plasmids. The methods includes steps of: providing single strand templates of the target nucleic acid to be amplified;

annealing of oligonucleotide primers to the templates; extending the oligonucleotide-template partial duplex in the presence of deoxynucleotide by a DNA polymerase; separating the duplex region of the DNA molecule with a helicase preparation; and repeating the above steps. The aforementioned steps may proceed in certain orders or simultaneously. Two primers flanking the sequence-of-interest are used to define the borders of the target sequence. Additional pair(s) of primers can be included to improve the yield.

In preferred embodiments, the DNA polymerase, the DNA helicase, and the SSB come from T7-like phage such as T7, T3, phiI, phiII, H, W31, gh-1, Y, All22, SP6 (Studier, *Virology* 95:70-84 (1979)).

In an embodiment of the invention, methods were described to produce high quality of T7 gene 4B helicase and T7 gene 2.5 SSB.

In additional embodiments of the invention, the DNA polymerase is preferably a processive polymerase which lacks 5′ to 3′ exonuclease activity and possesses strand displacement activity. The DNA polymerase may contain modifications or mutations which minimize its exonuclease activity or enhance its processivity, polymerization speed, or strand displacement activity. Plurality of DNA polymerases and/or accessory proteins may be included to improve the amplification.

In an embodiment of the invention, a helicase preparation may include a DNA helicase and accessory proteins for the helicase.

In an embodiment of the invention, the helicase preparation may include a single helicase or a plurality of helicases. The helicase or helicases in the preparation may be selected from the class of 5' to 3' helicases and/or the class of 3' to 5' helicases. The helicase may be a hexameric helicase or a monomeric or dimeric helicase.

Additionally, the helicase preparation may include accessory proteins for the helicase and the accessory protein may be a single strand DNA binding protein such as T7 gene 2.5 protein.

In an embodiment of the invention, the helicase preparation includes a nucleotide triphosphate (NTP) or deoxynucleotide triphosphate (dNTP) for example, adenosine triphosphate (ATP), deoxythymidine triphosphate (dTTP) or deoxyadenosine triphosphate (dATP). A suitable concentration for the energy source is in the range of about 0.1-50 mM.

In additional embodiments of the invention, the target nucleic acid may be either a single stranded circular nucleic acid or a double stranded circular nucleic acid or a culture containing circular DNAs. When the nucleic acid is double stranded or in a culture, it may be denatured by heat or helicases to provide single strand templates for amplification. In addition, the target nucleic acid may have a size in the range of about 50 bp to 500 kb.

Embodiments of the invention include amplification of a specific sequence from pure circular DNA samples and a culture containing circular DNAs. More importantly, it includes the amplification of the whole circular DNA from pure circular DNA samples and a culture containing circular DNAs. More specifically, a method is provided for a whole plasmid amplification. The amplification products can be used directly for sequencing, enzyme digestion, cloning, and other applications.

In additional embodiments of the invention, amplification may be isothermal and may be accomplished in the range of about 15°C-75°C, preferably at room temperature (25°C).

An embodiment of the invention features a method for selectively amplifying a target nucleic acid and/or the whole sequence of a DNA molecule, comprising preparing a helicase preparation containing a helicase(s), an NTP or dNTP, a buffer, wherein the buffer of Tris-acetate or Tris-HCl providing a pH in the range of about pH 5.5- 10.0, and a concentration of NaCl or KCl in a concentration range of 0-200 mM and optionally a single stranded binding protein(s) and/or an accessory protein(s); adding a target nucleic acid in varying concentrations or copy number, oligonucleotide primers and a DNA polymerase to the helicase preparation; incubating the mixture at a temperature between about 15°C and 75°C for up to several days; and analyzing the amplified DNA to determine whether amplification has occurred. Composition of the reaction mixture, conditions of the reaction and concentration of the reactants can be varied within certain

ranges provided herein to identify the optimum conditions for helicase-mediated plasmid amplification.

In an additional aspect, different approaches can be implemented to enable the current system to amplify from linear DNA templates. For example, linear DNA fragments can be converted into circular DNA molecule by a DNA ligase, generating circular DNAs which can then be amplified by the c-HDA method.

In an additional embodiment, c-HDA amplification may work as a detection system when coupled with other technology such as padlock probes (Nilsson et al., Science 265: 2085-2088 (1994)).

In an additional embodiment, c-HDA amplification may work as a reporting system to amplify circular DNA attached to antibodies in immunoassays and to improve the sensitivity of antibody-based detection system (Schweitzer at al., *Proc. Natl. Acad Sci. USA* 97:10113-10119 (2000)).

DNA helicases are used to separate two complementary DNA strands, generating single-stranded templates for the purpose of in vitro isothermal DNA amplification (US patent application No.10/665,633). This isothermal DNA amplification technology is named Helicase Dependent Amplification (HDA). It is capable of amplifying a specific target DNA sequence over a million fold by combining the unwinding activity of a helicase with the polymerization activity of a DNA polymerase.

Embodiments of the invention describe methods for amplifying circular DNA molecules using a modified Helicase-Dependent Amplification platform. The method of using a helicase and a DNA polymerase to amplify circular DNAs is here named circular- Helicase-Dependent Amplification (or c-HDA). By including a pair of specific primers, c-HDA can amplify not only the whole input DNA from a circular DNA template, but also a specific target sequence defined by the primers from the circular DNA template, which provides several additional advantages over existing methods such as Templiphi™ kit (Amersham Biosciences, Piscataway, NJ).

Embodiments of the invention have certain advantages over existing amplification methodologies which include:

(a) The target sequence is preferentially amplified over the plasmid backbone sequence providing extra material for downstream sequencing and analysis.

(b) The amplification product from a positive clone which harbors plasmids containing an insert of interest, shows a specific target fragment in addition to high molecular weight repeat units when separated by agarose gel. This can eliminate an extra step for screening clones containing inserts, for example by restriction enzyme digestion.

(c) Since helicase can separate DNA enzymatically, the initial heat-denaturation step required by Templiphi™ system can be omitted. Thus the entire reaction can be performed at a single temperature, which further reduces the reaction steps.

A helicase-mediated amplification technology (c-HDA) is described here for amplifying the whole circular DNA molecule as well as a specific target sequence within the circle. The mechanism of the amplification involves helicase-dependent rolling circle amplification as shown in FIG. 1. In the figure, the leading strand is designated as (+) strand and the lagging strand is designated as (-) strand. Amplification starts from a sense primer annealed to its complementary region of a (-) strand (FIG. 1, Step 1) and the primer is extended by a replisome which includes a DNA polymerase, a DNA helicase, and possibly additional accessory proteins such SSB (Step 2).

When replication reaches the 5'-end of the primer, a replisome displaces the primer along with the leading strand extended from the primer, initiating the continuing synthesis of the leading strand (Step 3). Concatemers of the templates are synthesized, which provides multiple priming sites for the reverse primers to anneal to (Step 4). Multiple rounds of strand-displacement synthesis by replisome produce specific target DNA fragments defined by two primers and multimers of the circular DNA.

The currently available Templiphi™ kit from Amersham Biosciences utilizes phi29 DNA polymerase to amplify the whole plasmid.

In one embodiment of the invention, a c-HDA system has been described which utilizes a T7 gene 4B helicase. The advantages of using a T7gene 4B helicase in c-HDA include the following:

(a) The amplification product from T7 c-HDA system can be easily handled, digested, and cloned. Second, because specific primers are used, contaminant DNAs in the sample are less a concern. More importantly, a specific fragment defined by the primers are also amplified in addition to the whole plasmid amplification. Therefore a single c-HDA reaction can serve two purposes: screening for positive clones which display the specific target fragment without restriction enzyme digestion and generating enough DNA for sequencing or cloning.

(b) The c-HDA system has the ability to amplify long specific target from a large circular DNA. As shown in FIG. 2 and FIG 7, long multimers of plasmids were above 40 kb and some products remained in the well. Therefore, a c-HDA reaction can isothermally amplify long target sequences of DNA (longer than 2000 nucleotides particularly, greater than 10,000 nucleotides, more particularly as much as about 100,000 nucleotides) and can amplify target sequences at one temperature for the entire process.

(c) In addition to the large circular DNA such as a large plasmid DNA, the c-HDA system also has the ability to amplify small circular DNA such as a circular oligodeoxynucleotide between 50 nucleotides and 300 nucleotides, ideally between 75 nucleotides and 150 nucleotides. The small circular DNA can be tagged to a molecule such as an antibody. The use of DNA amplification for the detection of antibodies bound to an antigen has been documented previously. In immuno-PCR, a unique DNA sequence tag is associated with a specific

antibody using streptavidin-biotin interactions (Sano et al., *Science* 258:120-122(1992)). Antibodies bound to antigens are then detected by PCR amplification of the associated DNA tag. Alternatively, a circular DNA can be tagged to an antibody and c-HDA can be used as a way of amplification instead of PCR.

In embodiments of the invention, an HDA preparation that includes T7 gene 4B helicase, T7 gene 2.5 SSB, and T7 sequenase is provided. T7 gene 4 protein is a hexameric replicative helicase which contains both a primase activity and a 5′ to 3′ helicase activity (Lechner and Richardson, *J. Biol. Chem*. 258:11185-11196 (1983)). The amino-terminal truncated version of gene 4 protein, T7 gene 4B protein (T7 4B helicase), only contains DNA helicase activity. T7 helicase unwinds DNA at a rate of 300 bp/sec with high processivity (Kornberg and Baker, DNA Replication, W.H. Freeman and Company, 2nd edit. 1992).

T7 gene 2.5 protein is a single strand DNA binding protein that has high homologous annealing capability (Yu and Masker, J. Bacteriol. 183: 1862-1869 (2001)). T7 SSB interacts with both T7 DNA polymerase and T7 gene 4 protein and stimulates both polymerase activity and helicase activity (Kim et al., *J. Biol. Chem*. 267:15032-15040 (1992); Notarnicola et al., J Biol. Chem. 272:18425-33 (1997)).

T7 DNA polymerase is composed with two polypeptides, one the T7 gene 5 protein and the other is the E. coli thioredoxin. Together they form a tightly associated complex conferring a high processivity to the polymerization reaction.

The T7 DNA polymerase can polymerize more than 70 kb in one binding event at a speed of about 300 nt/sec (Kornberg and Baker, DNA Replication, W.H. Freeman and Company, 2nd edit. 1992). The wild type T7 polymerase has high fidelity in DNA replication with an error rate of $1.5x10^5$ (Korpela et al., Nuc. Acid. Res. 19: 4967-4973 (1991)). The 3' to 5' exonuclease activity of T7 DNA polymerase (within gene 5 protein) can be inactivated selectively by reactive oxygen species or substitutions of key amino-acid residues in exonuclease active site (Tabor and Richardson, J. Biol. Chem. 264:6647-6658 (1987)). This exonuclease deficient form of the T7 DNA polymerase was name as modified T7 DNA polymerase (Tabor and Richardson, J. Biol. Chem. 264:6647-6658 (1987)) and is commercially available as Sequenase™ version 2.0 (USB Corporation, Cleveland, Ohio). Although the modified T7 DNA polymerase has lower/or no 3'-5' exonuclease activity which results in lower fidelity in DNA synthesis, it can initiate strand-displacement synthesis at a nick.

In certain circumstances, it may be desirable to utilize a plurality of different helicase enzymes and/or a plurality of different polymerases in an amplification reaction. The use of a plurality of helicases may enhance the yield and length of the amplification products under certain conditions where different helicases coordinate to increase the efficiency of the unwinding of duplex nucleic acids. For example, a helicase with low processivity which is able to melt blunt-ended DNA may be combined with a second helicase that has great

processivity but requires single-stranded tails for the initiation of unwinding. In this example, the first helicase initially separates the blunt ends of a long nucleic acid duplex generating 5′ and 3′ single-stranded tails and then dissociates due to its limited processivity. This partially unwound substrate is subsequently recognized by the second helicase, which continues the unwinding process with superior processivity. Similarly, a plurality of DNA polymerases may combine a polymerase with high processivity and a polymerase with stronger strand displacement activity. Or a polymerase with low fidelity can be combined with a polymerase with higher fidelity.

Generally, oligonucleotide primer pairs suitable for amplification are short synthetic oligonucleotides, for example, having a length of more than 10 nucleotides and less than 50 nucleotides. Design of primers involves various parameters such as melting temperature, primer length and GC content (Kampke et al., *Bioinformatics* 17:214-225 (2003)). Primers with perfect match to the target sequence are preferred. However, primers may include sequences at the 5' end which are non complementary to the target nucleotide sequence(s). Alternatively, primers may contain nucleotides or sequences throughout that are not exactly complementary to the target nucleic acid. Primers may represent analogous primers or may be non-specific or universal primers for use in HDA as long as specific hybridization can be achieved by the primer-template annealing at a predetermined temperature.

The primers may include any of the deoxyribonucleotide bases A, T, G or C and/or one or more ribonucleotide bases, A, C, U, G and/or one or more modified nucleotide (ribonucleotide or deoxyribonucleotide) wherein the modification does not prevent hybridization of the primer to the nucleic acid or elongation of the primer or denaturation of double stranded molecules. Primers may be modified with chemical groups such as phosphorothioates or methylphosphonates or with non nucleotide linkers (EPA 88 308 766 0) to enhance their performance or to facilitate the characterization of amplification products.

To make the detection of amplification easier, the primers may be subject to modification, such as fluorescent or chemiluminescent-labeling, and biotinylation. Other labeling methods include radioactive isotopes, chromophores and ligands such as biotin or haptens that can be readily detected by reaction with labeled forms of their specific binding partners e.g. streptavidin and antibodies, respectively.

More primers can be included to improve the yield of the specific amplification products and the whole circular DNA. Additional primers may sit next to the first pair of primers or keep a certain distance.

Chemical reagents, such as denaturation reagents including urea and dimethyl-sulfoxide (DMSO) can be added to the c-HDA reaction to partially denature or de-stabilize the duplex DNA. Molecular crowding reagents, such as polyethylene glycol (PEG), may be included to improve the efficiency and yield of the amplification. Protein stabilizing

reagents such as trehalose may be included to improve the amplification

The following factors may optionally be added to the amplification mix- NTP or dNTP regeneration system and topoisomerase which can release the tension in the duplex DNA (Kornberg and Baker, DNA Replication, W.H. Freeman and Company, 2nd edit. 1992).

Definitions

Certain terms employed in the specification, examples and appended claims are collected here.

The term "Nucleic acid" refers to double stranded or single stranded DNA, RNA molecules or DNA/RNA hybrids. Those molecules which are double stranded nucleic acid molecules may be nicked or intact. The nucleic acid may be isolated from a variety of sources including the environment, food, agriculture, fermentations, biological fluids, biological tissue samples or cells. Circular DNAs may occur naturally or may be produced by in vitro ligation or chemical synthesis. Any of the above described nucleic acids may be subject to modification where individual nucleotides within the nucleic acid are chemically altered (for example, by methylation). Modifications may arise naturally or by *in vitro* synthesis.

The term "target nucleic acid" refers to a whole or part of nucleic acid to be selectively amplified and which is defined by 3' and 5' boundaries. The target nucleic acid may also be referred to as a fragment or sequence that is intended to be amplified. The size of the target nucleic acid to be amplified

may be, for example, in the range of 50 bp to 500 kb. The target nucleic acid may be contained within a longer double stranded or single stranded nucleic acid.

The terms "melting", "unwinding" or "denaturing" refer to separating all or part of two complementary strands of a nucleic acid duplex.

The terms "hybridization" and "annealing" refers to binding of an oligonucleotide primer to a region of the single-stranded nucleic acid template under the conditions in which a primer binds only specifically to its complementary sequence on one of the template strands, not other regions in the template. The specificity of hybridization may be influenced by the length of the oligonucleotide primer, the reaction temperature, the ionic strength, and the pH.

The term "primer" refers to a single stranded nucleic acid capable of binding to a single stranded region on a target nucleic acid to facilitate polymerase dependent replication of the target nucleic acid.

The term "accessory protein" refers to any protein capable of stimulating a helicase activity or a polymerase activity. For example, T7 SSB stimulates the T7 helicase activity and T7 polymerase activity; therefore T7 SSB is referred to as an accessory protein in the T7-based c-HDA system. Thioredoxin is a processivity cofactor for T7 polymerase and can be considered as an accessory protein.

The term "cofactor" refers to small-molecule agents that are required for the helicase unwinding activity. Helicase cofactors include nucleoside triphosphate (NTP) and

deoxynucleoside triphosphate (dNTP) and magnesium (or other divalent cations). For example, dTTP (deoxythymidine triphosphate) may be used as a cofactor for T7 Gp4B helicase at a concentration in the range of 0.1-100 mM and preferably in the range of 1 – 20 mM.

The term "helicase" refers here to any enzyme capable of unwinding a double stranded nucleic acid enzymatically. Any helicase that translocates along DNA or RNA in a 5′ to 3′ direction or in the opposite 3′ to 5′ direction may be used in present embodiments of the invention. Helicases are found in almost all organisms and can be obtained from prokaryotes, viruses, archaea, and eukaryotes. Alternally helicases can be recombinant forms of naturally occurring enzymes as well as analogues or derivatives having the specified activity. Examples of naturally occurring DNA helicases (see Kornberg and Baker, DNA Replication, W.H. Freeman and Company, 2nd edit. 1992) include *E. coli* helicase I, II, III, & IV, Rep, DnaB, PriA, PcrA, T4 Gp41 helicase, T4 Dda helicase, T7 Gp4B helicases, SV40 Large T antigen, Rho helicase, and yeast RAD. Examples of helicases for use in present embodiments may also be found at the following web address: http://blocks.fhcrc.org (Get Blocks by Keyword: helicase).

The unwinding activity of a helicase during DNA amplification relies on a cofactor which provides an energy source such as a nucleotide triphosphate (NTP) or deoxynucleotide triphosphate (dNTP) and the activity may be further enhanced with additional reagents. The additional reagents include one or more selected from the following: an

additional helicase or helicases, an accessory protein, a single strand binding protein (SSB), small molecules, chemical reagents and a buffer.

The helicase plus an energy source and one or more additional reagents as specified above are collectively referred to as a "helicase preparation". For example, the unwinding activity of T7 4B helicase utilizes dTTP as an energy source and its activity may be enhanced with an accessory protein, for example, T7 Gp2.5.

The term "c-HDA system" has been used herein to describe a group of interacting elements for performing the function of amplifying nucleic acids according to the Circular Helicase-Dependent Amplification method described herein. The c-HDA system includes a helicase preparation, a polymerase and optionally other enzymes or proteins that stimulate the amplification. For example, the T7-based c-HDA system may be constituted by mixing together a T7 helicase preparation (T7 Gp4B helicase, T7 Gp2.5 SSB, and dTTP) and T7 Sequenase. The reaction may be optimized by substitution, addition, or subtraction of elements within the mixture. This process utilizes two oligonucleotide primers, each hybridizing to the borders of the target sequence. More pairs of primers may be included to improve the amplification.

"Isothermal amplification" refers to amplification which occurs at a single temperature. This does not include the single brief time period (less than 15 minutes) at the initiation of amplification which may be conducted at the same

temperature as the amplification procedure or at a higher temperature.

EXAMPLES

Example I
CLONING AND PURIFYING T7 4B HELICASE AND ITS ACCESSORY PROTEIN T7 2.5 SSB

1. Cloning the genes encoding T7 4B helicase and 7 gene 2.5 single strand binding protein (SSB).

Genes encoding T7 4B helicase (GenBank accession number: AAP33931) and T7 2.5 SSB (GenBank accession number: AAP33924) were cloned using the Impact™ system (New England Biolabs, Inc. (NEB), Beverly, MA). T7 gene 4B was amplified from T7 DNA (NEB) using primer 4B51 (SEQ ID NO:1) and 4B31 (SEQ ID NO:2). T7 gene 2.5 was amplified from T7 DNA using primer 2551 (SEQ ID NO:3) and 2531 (SEQ ID NO:4). PCR products were digested with SapI and NdeI and introduced into plasmid pTYB1 (NEB) to give pTYB4B or pTYB25. Plasmids containing the correct sequence were transformed into ER2566 cells (NEB). ER2566 cells containing either pTYB4B or pTYB25 were grown at 37°C in LB media supplemented with 50 µg/ml carbenicilin. When OD_{600} reached ~ 0.7, protein expression was induced with 0.3 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After an overnight incubation at 15°C, cells were harvested for purification.

2. T7 4B protein and T7 2.5 protein purification

All procedures were performed at 4°C. Cells expressing 4B or 2.5 protein from a 3-liter culture were resuspended in

60 ml column buffer (20mM Tris pH 8.0, 1mM EDTA, 500 mM NaCl) and were broken by sonication. After centrifugation, the clarified extract was loaded on a 15-ml chitin bead column pre-equilibrated with 150 ml of column buffer. The column was washed with 300 ml of column buffer. Induction of self-cleavage was conducted by flushing the column with 3 column volumes (45 ml) of cleavage buffer (column buffer + 50 mM dithiothreitol (DTT)). The cleavage reaction was carried out at 4°C for 64 hours in the cleavage buffer. The protein was eluted with the column buffer. The positive fractions were pooled and diluted to a final salt concentration of 50mM NaCl. Diluted proteins were loaded on a 1 ml-SourceQ column (Pharmacia, Piscataway, NJ) which had been pre-equilibrated with buffer A (20 mM Tris.Cl, pH7.5, 50 mM NaCl, 0.1mM EDTA, 1mM DTT, 5% glycerol). Fractions containing target proteins were pooled and diluted to 50mM NaCl. Diluted proteins were loaded on a 1 ml-Heparin column (Pharmacia, Piscataway, NJ) which had been pre-equilibrated with buffer A (20 mM Tris.Cl, pH7.5, 50 mM NaCl, 0.1mM EDTA, 1mM DTT, 5% glycerol). Fractions containing gene 4B protein free of nuclease contamination was dialyzed against buffer (20 mM Tris.Cl, pH7.5, 100 mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol). Fractions containing gene 2.5 protein free of nuclease contamination was dialyzed against buffer (20 mM Tris.Cl, pH7.5, 150 mM NaCl, 0.1mM EDTA, 1mM DTT, 50% glycerol). Proteins were stored at –70–C.

EXAMPLE II

AMPLIFICATION OF CIRCULAR DNA MOLECULES USING C-HDA

c-HDA can be used to amplify circular DNA such as circular oligodeoxynucleotides and circular DNA plasmids. The amplification of circular DNA molecules by c-HDA can be used as tools in molecular biology applications. For example, the small circular DNA can be tagged to a molecule such as an antibody. The use of amplification of a circular oligonucleotide to increase the sensitivity of immuno detection has been documented previously (Schweitzer et al., Proc. Natl. Acad. Sci. USA 97:10113-10119 (2000)). C-HDA can also be used to amplify a large circular DNA such as a plasmid DNA from purified plasmid DNA or from a colony of bacterial cells. In this example we disclose a helicase-based in vitro DNA amplification method which can be used to amplify circular nucleic acid molecules. More specifically, we describe a helicase-mediated plasmid amplification method which depends on the helicase and uses two specific primer.

A pCR2.1 (Invitrogen, Carlsbad, CA) derivative plasmid pREP containing *E.coli rep* helicase (GenBank accession number U00096) was used as a target template. Oligos S1224 and S1233 (NEB products) flanking the *rep* insert were used as primers. A 50 µl reaction was set up by mixing 5 µl of 10X c-HDA Buffer (350 mM Tris-Acetate pH7.5, 11 mM Mg Acetate, 50mM DTT), 10 ng pREP, 20 pmole S1224, 20 pmole S1233, 50 nmole dNTP, 500 nmole dTTP, 200ng T7 4B protein,

6 µg T7 2.5 protein, and 1 unit T7 sequenase (USB, Cleveland, OH). The reaction was incubated at 25°C overnight. Five µl reaction products was analyzed on a 1% agarose gel containing ethidium bromide (FIG. 2A). A DNA fragment about 2.3 bp was observed (FIG. 2A, lane 1), matching the size predicted from the target sequence. In addition, higher molecular weight DNA bands corresponding to multiple repeats of plasmid DNA plus insert were also observed (FIG. 2A, lane 1). The 2.3 kb amplification products were later sequenced and the sequencing results confirmed that the product was derived from the *Rep* gene.

The requirement of each T7 protein was investigated. When one of the three T7 proteins was left out, there was no amplification product (FIG. 2A, lanes 2, 3, and 4), indicating that all three proteins are required for the c-HDA reaction. Similarly, when both helicase and SSB were left out, no amplification was detected (FIG. 2A, lane 5). When T7 SSB was replaced with equal amount of T4 SSB, there was no amplification, indicating that a close coordination among the three T7 proteins is crucial for the c-HDA amplification. With a prior denaturation step, sequenase alone can synthesize low amount of multimers of the plasmid (data not shown); however, all three proteins must be present to amplify the specific 2.3 kb fragment.

To analyze the amplification products of higher molecular weight, a pulse-field gel electrophoresis was conducted. In Figure 2. lane 1 shows the original plasmid and lane 2 shows

the amplification product. A ladder corresponding to multiple repeat units of 6-kb plasmid was observed. The smallest band b1 is about 8.3 kb, which matches the predicted size of plasmid pREP (6 kb) plus the insert (2.3 kb). The next band b2 is about 14.3 kb, which also matches the predicted size (2 x 6 kb + 2.3 kb).

In other isothermal amplification methods, a prior denaturation step is either required for the amplification or can improve the final yield. To investigate the effect of a prior denaturation step on c-HDA reactions, two reactions were carried out: one c-HDA reaction was incubated at 25°C for the entire reaction; in the other reaction plasmid templates and primers were first denatured at 95°C and then mixed with other c-HDA components at 25°C. As shown in Figure 2C, the yield of amplification was slightly higher in the 2-step reaction (lane 2) than the one-step reaction (lane 1). However, the difference was quite small.

Restriction enzyme digestions were conducted on the amplification products to further prove that tandem repeats of the plasmid containing the target fragment were produced. Acc65I, SacI, and XhoI have a unique site on pREP (FIG. 3A). When pREP was digested with either Acc65I or SacI, pREP was linearized as a 6-kb fragment (FIG. 3B, lanes 1 and 2). When pREP was digested with XhoI and SacI, pREP was cleaved into two pieces, a 2.2-kb fragment and a 3.8-kb fragment (FIG. 3B, lane 3). When the amplification product was digested with either Acc65I or SacI, the specific 2.3 kb fragment was observed in addition to the linearized 6 kb

plasmid (FIG. 3B lanes 5 and 6). When the amplification product was digested with XhoI and SacI, a 3.8 kb and the 2.2 kb fragments were produced, indicating that 2.3 kb and 6 kb fragments by a single SacI digestion was further cleaved into 2.2 kb and 3.8 kb by XhoI (FIG. 3B, lane 7).

To test whether the reaction products can be used directly for sequencing on an ABI sequencer, 1 µl product was used directly for sequencing by S1224 (Figure 4A) or a *Rep*-specific primer (Figure 4B). The sequencing reactions gave nice peaks with low background, indicating that the c-HDA products can be used as sequencing templates without any cleanup step. The sequencing reactions offered confident readings up to 600 bp (data not shown).

To test whether both primers are needed for the amplification, c-HDA reactions were carried out with one primer or both primers using pREP plasmid templates. As shown in Figure 5, in the presence of two primers, both the specific 2.3 kb fragment and larger fragments were amplified (lane 1). When only one primer is used (either S1224 or S1233), no corresponding amplification products were observed in the gel (Figure 5, lanes 2 and 3). However, there was small amount of DNA remained in the well, which is probably the single stranded concatemers of the plasmid produced by a linear RCA amplification.

EXAMPLE III
AMPLIFICATION OF PLASMID DNA DIRECT FROM A BACTERIAL COLONY

Plasmid DNA has been widely used as a tool in molecular biology research. A gene can be cloned into a plasmid. Traditionally, plasmid DNA is amplified in vivo by purified by growing bacterial cells. The plasmid DNA was then purified from bacterial cells by various methods. To circumvent the need of cell culturing, A Phi29 DNA polymerase-based in vitro plasmid DNA amplification method has been described previous (Nelson et al., *Biotechniques* Suppl: 44-7 (2002)). In this example we disclose a helicase-based method to amplify plasmid DNA directly from a single bacterial colony.

To test whether c-HDA can be used to amplify DNA plasmid directly from E. coli colonies, plasmid pREP was transformed into *E.coli* ER2502 (NEB). A colony was resuspended in 20 µl resupsension buffer (20 mM Tris.Acetate pH7.5 buffer). A 5 µl resupsension buffer containing 1-5 µl colony suspension was incubated at 95°C for 3 min and cooled to 25°C. A 45 µl mix containing 1 unit sequenase (USB), 7 µg T7 SSB, 250 ng T7 4B helicase, 50 pmole dNTP, 500 nmole dTTP, 5 pmole S1224, 5 pmole s1233, and 1x c-HDA buffer was added to each tube and incubated at 25°C for 6 hrs. As shown in FIG. 6, the amplification product from a colony includes a 2.3 kb fragment and molecules of higher molecular weight (lanes 2 to 6), which is the same as that from pure plasmids (lane 1). The amplification product from a colony was used directly for sequencing using S1224 or *rep*-

specific primers. The sequencing results were good. This result suggests that c-HDA can be used to amplify plasmid DNA from a single E. coli colony and the amplified DNA can be used for sequencing and restriction digestion.

EXAMPLE IV
METHOD OF AMPLIFICATION OF A DNA PLASMID WITH A LARGE-SIZE INSERT.

To test whether T7-based c-HDA can amplify even longer target and plasmid, a pCR2.1 plasmid derivative pTOPO10k containing a 10-kb insert was used as a target template. Oligos S1224 and S1233 (NEB) flanking the insert were used as primers. A 50-µl reaction was set up by mixing 5 µl of 10X c-HDA Buffer, 20 ng pTOPO10k, 20 pmole S1224, 20 pmole S1233, 50 nmole dNTP, 500 nmole dTTP, 200 ng T7 4B protein, 6 µg T7 SSB, and 1 unit T7 sequenase. After an overnight reaction, 5 µl was separated on a 1% agarose gel and stained with ethidium bromide (FIG. 7A). A 10 kb DNA band and DNAs of larger size were detected on the gel.

When the product was separated by pulse-field gel electrophoresis, a ladder pattern of DNA fragments was observed (Figure 7B). The smallest band b1 is about 10 kb, which is the specific band. The next band b2 is about 24 kb, which matches the predicted size (14 kb of pTopo10k + 10 kb insert). Similar to the amplification products from pREP, the amplification products from pTopo10k can be cleaved into sizes of insert and plasmid by restriction enzyme digestion

(data not shown). The amplification products can also be sequenced directly (data not shown).

EXAMPLE V
AMPLIFICATION OF PLASMID DNA DIRECT FROM A BACTERIAL COLONY

Plasmid DNA has been widely used as a tool in molecular biology research. A gene can be cloned into a plasmid. Traditionally, plasmid DNA is amplified in vivo by purified by growing bacterial cells. The plasmid DNA was then purified from bacterial cells by various methods. To circumvent the need of cell culturing, A Phi29 DNA polymerase-based in vitro plasmid DNA amplification method has been described previous (Nelson et al., *Biotechniques* Suppl: 44-7 (2002)). In this example we disclose a helicase-based method to amplify plasmid DNA directly from a single bacterial colony.

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from pure plasmids (lane 1). The amplification product from a colony was used directly for sequencing using S1224 or *rep*-specific primers. The sequencing results were good. This result suggests that c-HDA can be used to amplify plasmid DNA from a single E. coli colony and the amplified DNA can be used for sequencing and restriction digestion.

EXAMPLE VI

DETECTION OF PATHOGENIC BACTERIA CONTAINING PLASMIDS USING C-HDA

Many pathogenic bacteria harbor circular DNA plasmids, which play important roles in pathogenesis and antibiotics resistance. For example, the large plasmid in Bacillus anthracis harbors anthrax toxin gene (Okinaka et al., 1999). Since the c-HDA method disclosed in this invention is able to amplify circular DNA molecules including plasmid, it can be used when combining with a DNA detection method to amplify and detect plasmids associated with specific pathogens. The following is a hypothetic example illustrating the application of c-HDA in detecting bacterial pathogens containing plasmid from specimens.

Specimens are treated to concentrate potential pathogens for example by centrifugation. The concentrated sample is then resuspended in a small volume of lysis solution that may include detergents, and/or enzymes to break cell-wall and cell-membrane of bacteria or bacterial spores. The details of bacterial spore lysis was described previously (Ryu et al., Miocrobiol. Immunol. 47:693-699 (2003)). Heat (95° -

100°C) may also be used to help broken the cells. The lysed specimens (say 10 µl) is combined with 40 µl of c-HDA mixture including T7 Sequenase, T7 SSB, T7 4B helicase, dNTP, 500 nmole dTTP, primers specific to the target sequence in the plasmid, and c-HDA buffer (detailed in Example III). Following the amplification, amplified products are analyzed by various methods, including agarose gel electrophoresis, or immuno-chromatographic strips (Matsubara AND Kure, Human Mutation, 22:166-172 (2003)), or realtime time-detection via fluorescence signals.

EXAMPLE VII
A KIT FOR AMPLIFICATION PLASMID DNA

The methods disclosed in embodiments of the invention may be incorporated into a kit. For example, a kit for amplification of plasmid DNA may be commercialized as the following novel composition. A c-HAD plasmid amplification kit may be composed of a colony suspension buffer, a c-HDA reaction buffer, a DNA polymerase, four dNTPs (dATP, dGTP, dCTP, dTTP), and a helicase preparation which may contains one or more DNA helicases, cofactor required for unwinding, and accessory proteins (if any) such single-stranded binding protein. The kit also includes a manual of how to perform plasmid DNA amplification according to Example II and III. The kit may also include a control plasmid and a pair of primers.

IN THE CLAIMS

What is claimed is:

1. A method for amplifying a predetermined portion of a circular nucleic acid, comprising:

(a) providing the circular nucleic acid;

(b) annealing to the circular nucleic acid, sequence-specific oligonucleotide primers flanking the predetermined portion;

(c) adding a DNA polymerase, a helicase preparation, and dNTPs;

(d) permitting rolling circle amplification on one nucleic acid strand and strand-displacement amplification on the complementary strand; and

(e) amplifying the predetermined portion of the circular nucleic acid.

ABSTRACT

This invention relates to a helicase-mediated amplification method for amplifying DNA from a circular nucleic acid template. This invention more particularly describes a process in which DNA helicases unwind double-stranded nucleic acid duplexes to facilitate a DNA polymerase in rolling circle amplification and/or strand displacement amplification. The amplification products include both a specific target fragment and concatemers of the circular DNA templates.

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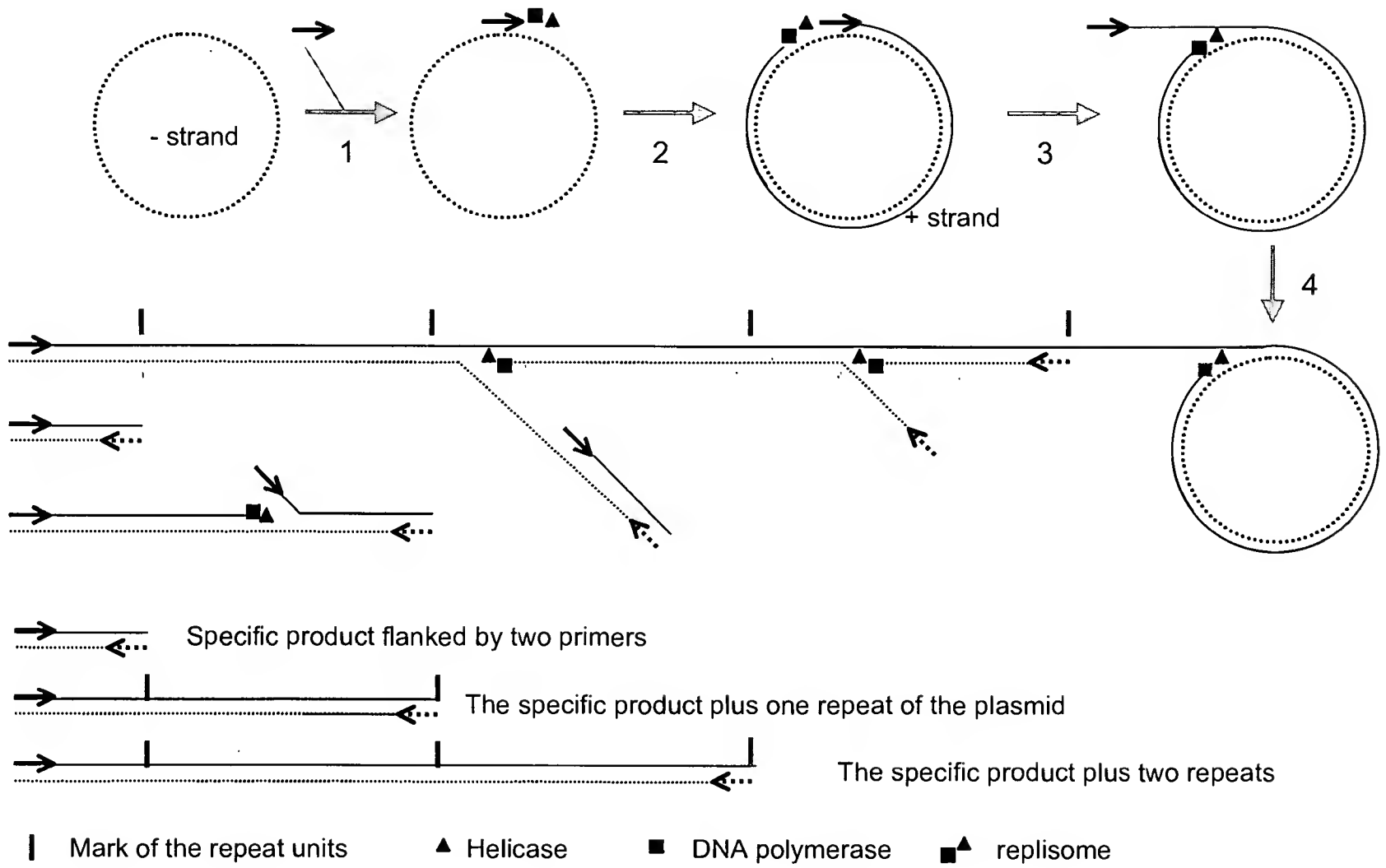

Figure 1. Schematic diagram of a c-HDA reaction. Step 1: Primer annealing to the - strand (shown in dashed circle); Step 2: primer extension; Step 3: Unwinding by a helicase displaces the + strand (shown in solid line); Step 4: continue displacement synthesis of the + strand, reverse primers binding to the + strand and initiates multiple displacement synthesis of the - strands.

Figure 2. Circular-HDA reactions from plasmid pREP.

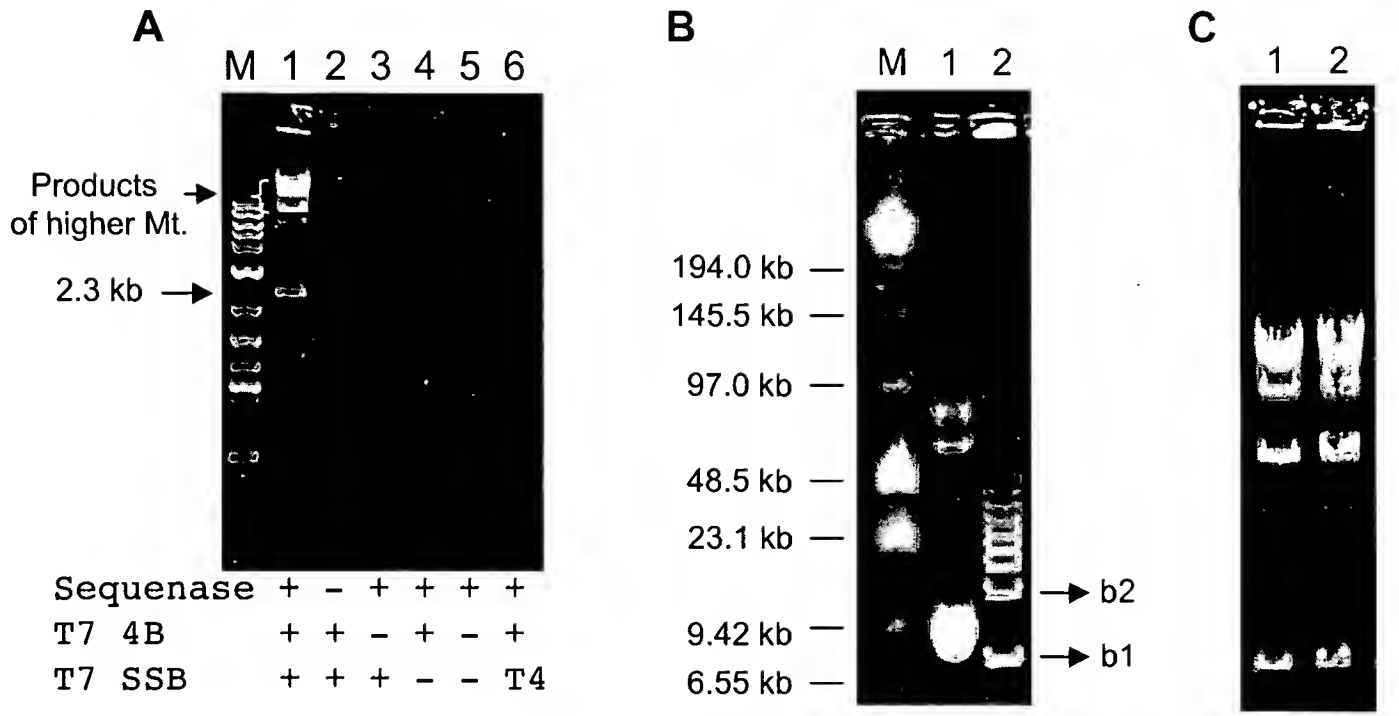

Figure 3. Restriction enzyme digestion on the c-HDA products.

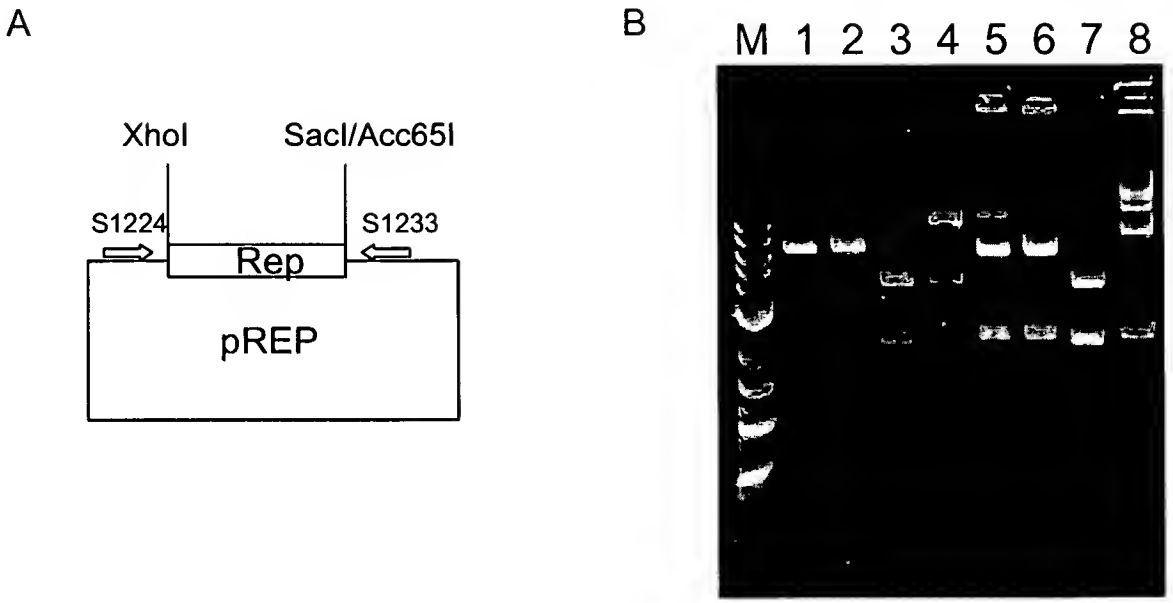

Figure 4. Direct sequencing reactions using c-HDA products.

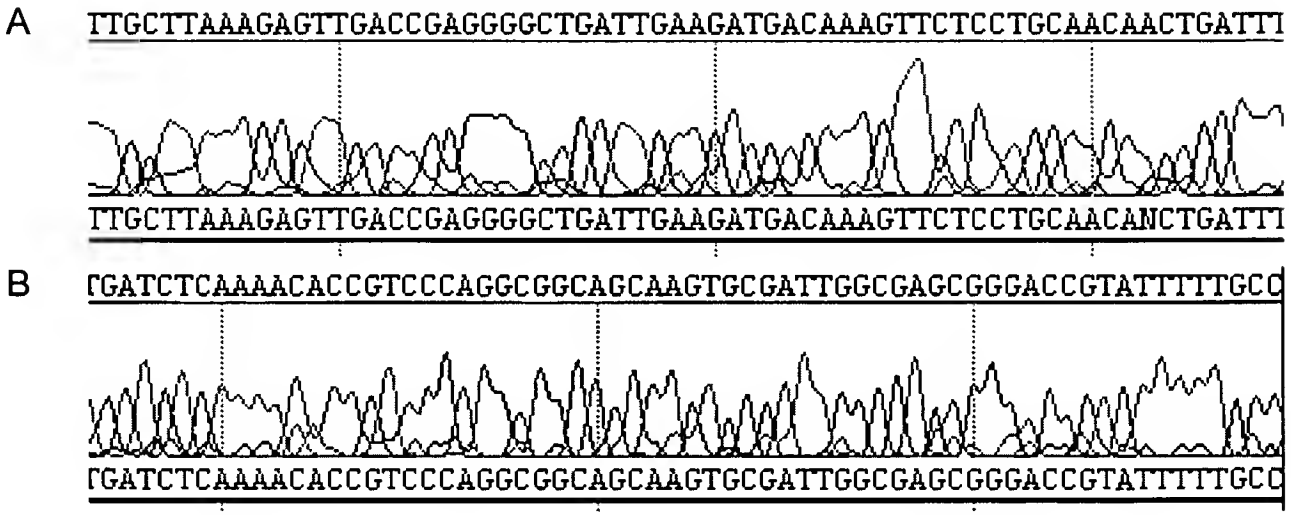

Figure 5. Expotential c-HDA amplification requires two primers.

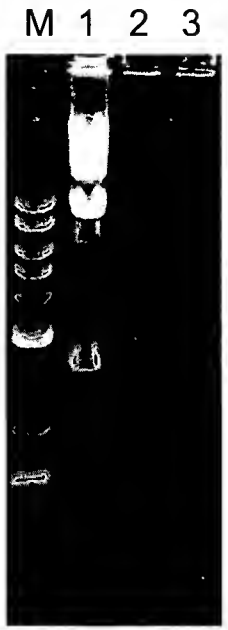

Figure 6. Circular-HDA from a colony containing pREP plasmid

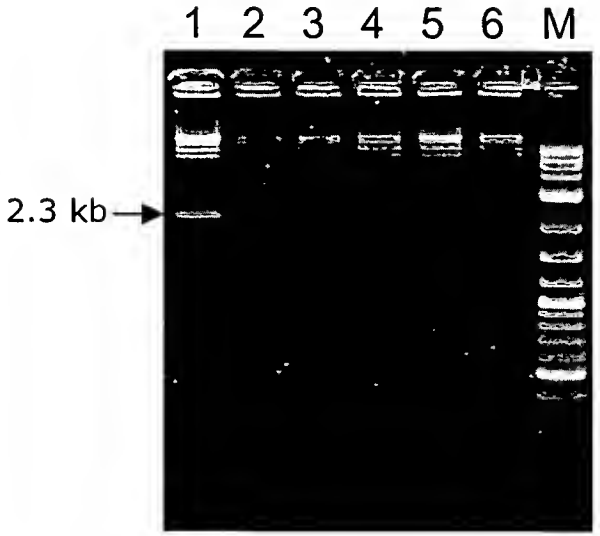

Figure 7. Amplification of a 10-kb fragment by c-HDA

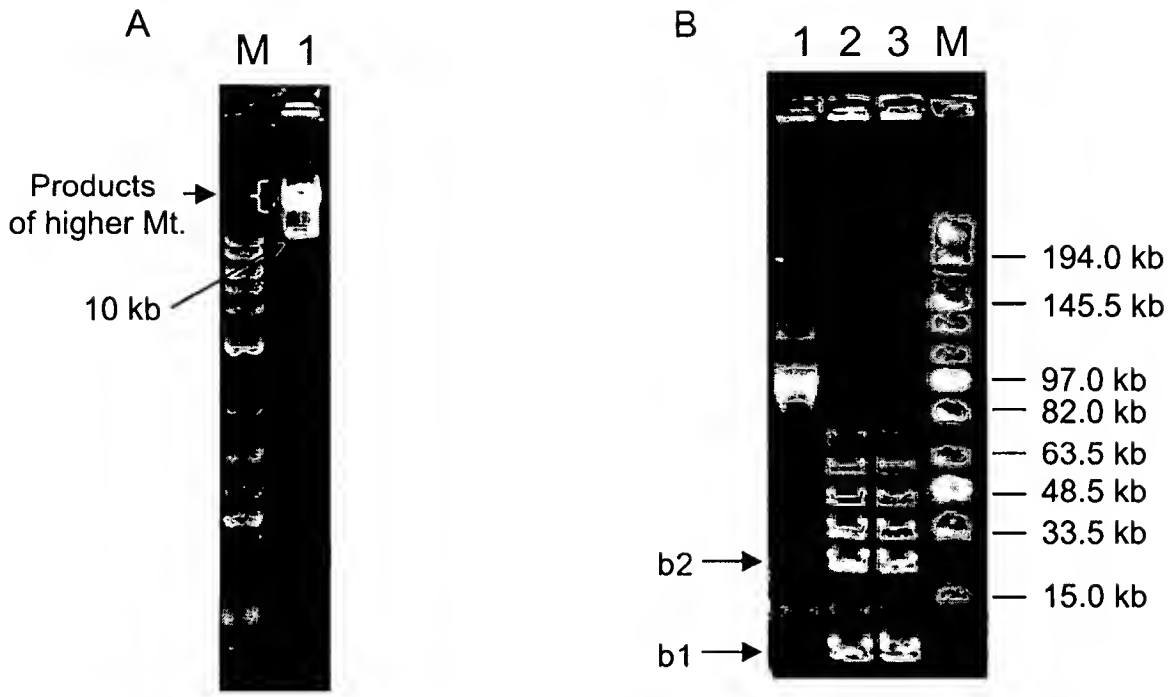

Figure 8.

Sequence Listing

1) Primer 4B51 (SEQ ID NO:1)
5' ACCCTTTCATATGACTTACAACGTGTGGAACTTC 3'

2) Primer 4B31 (SEQ ID NO:2)
5' GGAAATGCTCTTCCGCAGAAGTCAGTGTCGTTGGACC 3'

3)) Primer 2551 (SEQ ID NO:3)
5'ACCCTTTCATATGGCTAAGAAGATTTTCACCTCTGCGCTGGGTACCGCTGAACCTTACGCT
TACATCGCCAAGCCGGACTACGGCAACGAGGAGCGTGGCTTTGGGA3'

4)) Primer 2531 (SEQ ID NO:4)
5' GGAAATGCTCTTCCGCAGAAGTCTCCGTCTTCGTCTG 3'